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The A78V Mutation in the Mad3-like Domain of *Schizosaccharomyces pombe* Bub1p Perturbs Nuclear Accumulation and Kinetochore Targeting of Bub1p, Bub3p, and Mad3p and Spindle Assembly Checkpoint Function

Sheila Kadura,[*] Xiangwei He,[†‡] Vincent Vanoosthuyse,[§] Kevin G. Hardwick,[§] and Shelley Sazer[*†||]

[†]Verna and Marrs McClean Department of Biochemistry and Molecular Biology and [*]Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX 77030; and [§]Wellcome Trust Centre for Cell Biology, University of Edinburgh, Edinburgh EH9 3JR, United Kingdom

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During mitosis, the spindle assembly checkpoint (SAC) responds to faulty attachments between kinetochores and the mitotic spindle by imposing a metaphase arrest until the defect is corrected, thereby preventing chromosome missegregation. A genetic screen to isolate SAC mutants in fission yeast yielded point mutations in three fission yeast SAC genes: *mad1, bub3,* and *bub1.* The bub1-A78V mutant is of particular interest because it produces a wild-type amount of protein that is mutated in the conserved but uncharacterized Mad3-like region of Bub1p. Characterization of mutant cells demonstrates that the alanine at position 78 in the Mad3-like domain of Bub1p is required for: 1) cell cycle arrest induced by SAC activation; 2) kinetochore accumulation of Bub1p in checkpoint-activated cells; 3) recruitment of Bub3p and Mad3p, but not Mad1p, to kinetochores in checkpoint-activated cells; and 4) nuclear accumulation of Bub1p, Bub3p, and Mad3p, but not Mad1p, in cycling cells. Increased targeting of Bub1p-A78V to the nucleus by an exogenous nuclear localization signal does not significantly increase kinetochore localization or SAC function, but GFP fused to the isolated Bub1p Mad 3-like accumulates in the nucleus. These data indicate that Bub1p-A78V is defective in both nuclear accumulation and kinetochore targeting and that a threshold level of nuclear Bub1p is necessary for the nuclear accumulation of Bub3p and Mad3p.

INTRODUCTION

During anaphase of mitosis, sister chromatids are separated by the mitotic spindle. The spindle assembly checkpoint (SAC) protects against genetic loss by preventing anaphase initiation until all chromosomes are properly attached to the spindle microtubules (for review see Wassmann and Benezra, 2001). Screens in *Saccharomyces cerevisiae* identified the first SAC genes based on the sensitivity of bub and mad mutants to microtubule disruption (Hoyt *et al.*, 1991; Li and Murray, 1991). *MPS1* was identified as required for both the SAC and spindle pole body duplication (Weiss and Winey, 1996).

The first two SAC genes in *Schizosaccharomyces pombe*, *mad2* and *mph1*, were identified based on their ability to promote a metaphase arrest and inhibit proliferation when overexpressed in wild-type cells and to render cells checkpoint-defective when mutated (He *et al.*, 1997, 1998). Fission yeast Mad2p was independently identified based on its interaction with the APC activator, Slp1p (Kim *et al.*, 1998).

Homologues of the yeast SAC proteins have been identified in higher eukaryotes (for review see Millband *et al.*, 2002). The SAC is not a linear genetic pathway, and it does not operate identically in all eukaryotes (for review see Bharadwaj and Yu, 2004), but a consensus model for SAC function is emerging (for review see Millband *et al.*, 2002). The checkpoint proteins exist in small subcomplexes, the composition of which is altered during SAC-induced mitotic arrest (for review see Millband *et al.*, 2002). Stabilization of a Bub1p-Mad1p-Bub3p complex in checkpoint-activated cells is required for checkpoint function (Brady and Hardwick, 2000). During checkpoint activation, the Mps1, Mad1, Mad2, Mad3, Bub1, and Bub3 checkpoint proteins localize to unattached kinetochores (for review see Cleveland *et al.*, 2003). The Mps1, Mad2, BubR1, and Bub3 proteins associate dynamically with kinetochores, whereas Mad1p and Bub1p bind more stably (Howell *et al.*, 2000, 2004; Shah *et al.*, 2004). Mad2p, Mad3p, and Bub3p impose a metaphase arrest by binding to the anaphase promoting complex (APC) and inhibiting its ubiquitin ligase activity (for review see Yu, 2002), thereby preventing the ubiquitination and subsequent degradation of critical cell cycle regulatory proteins by the 26S proteasome.

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[‡] Present address: Department of Human and Molecular Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030.

[||] Corresponding author. E-mail address: ssazer@bcm.tmc.edu.

Abbreviations used: SAC, spindle assembly checkpoint; Bub, budding uninhibited by benomyl; Mad, mitotic arrest deficient; NLS, nuclear localization signal; MBC, carbendazim; TBZ, thiabendazole; HU, hydroxyurea; YE, yeast extract; EMM, Edinburgh minimal media.

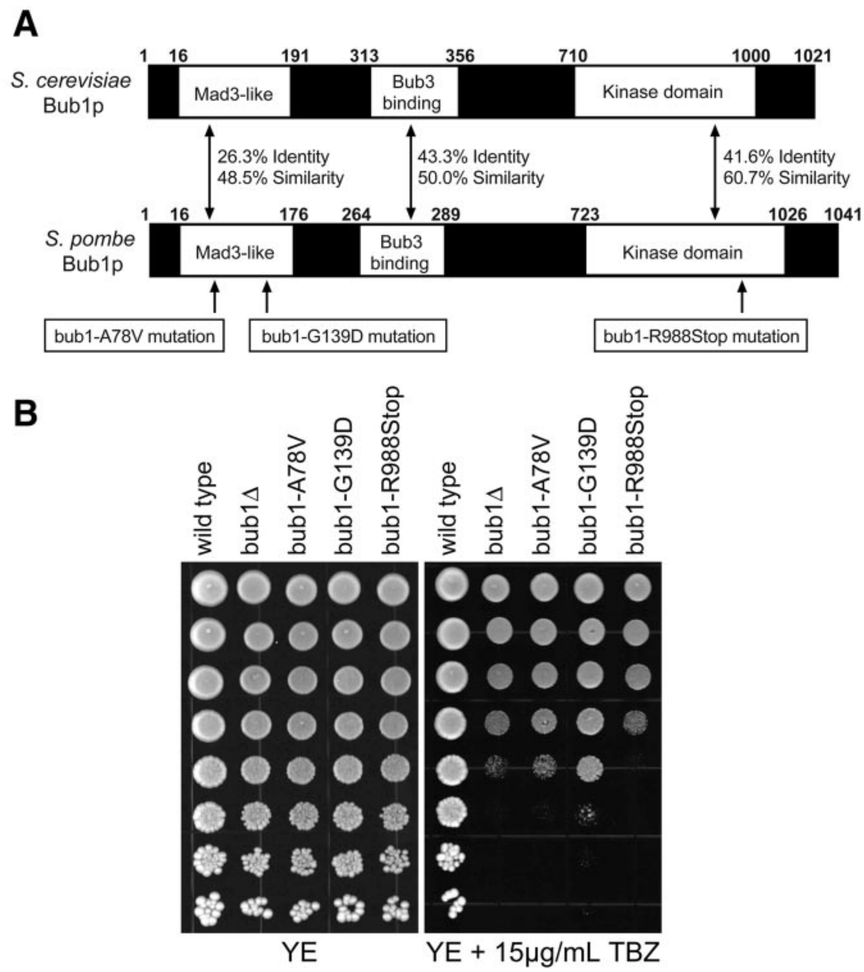


Figure 1. The *mph1* overexpression screen identified three novel *bub1* mutant alleles. (A) Comparison of the known domains in *S. cerevisiae* Bub1p and similar domains in *S. pombe* Bub1p. The bub1-A78V and bub1-G139D mutations occur in the conserved Mad3-like region. The bub1-R988Stop mutation introduces a premature stop codon in the protein kinase domain, which removes 82 amino acids from the carboxy terminus of Bub1p. (B) Cells were grown in rich medium and spotted onto plates in fivefold dilutions starting with 10^6 cells and grown at 32°C in the presence or absence of TBZ.

Bub1p is required for SAC function in mitosis (for review see Lew and Burke, 2003) and is essential for meiosis in fission yeast (Bernard *et al.*, 2001; Yamaguchi *et al.*, 2003) and flies (Basu *et al.*, 1999). The evolutionarily conserved Bub1p domain structure includes a kinase domain, a Bub3p-binding domain, and a Mad3-like region, the function of which is unknown (see Figure 1A; Taylor *et al.*, 1998; Hardwick *et al.*, 2000; Warren *et al.*, 2002). Bub1p kinase activity is required for efficient checkpoint function in fission yeast (Yamaguchi *et al.*, 2003), but its in vivo targets are unknown. Bub1p localizes to kinetochores in every mitosis (Taylor and McKeon, 1997; Bernard *et al.*, 1998; Jablonski *et al.*, 1998; Ouyang *et al.*, 1998; Basu *et al.*, 1999; Sharp-Baker and Chen, 2001; Gillett *et al.*, 2004) and regulates the kinetochore association of other SAC proteins (Basu *et al.*, 1998; Sharp-Baker and Chen, 2001; Millband and Hardwick, 2002; Gillett *et al.*, 2004; Johnson *et al.*, 2004).

The SAC can be activated by defects in microtubule/kinetochore interactions or by overexpression of some SAC components (for review see Bharadwaj and Yu, 2004). The cell cycle arrest due to *mph1* overexpression requires *mad2*, indicating that *mad2* acts downstream of *mph1* (He *et al.*, 1998). The observation that mad2Δ cells survive *mph1* overexpression also indicates that kinetochore/microtubule interactions are unperturbed. A genetic screen to isolate fission yeast strains that, like mad2Δ, are insensitive to *mph1* overexpression identified five novel mutant alleles of three checkpoint genes: *bub1*, *bub3*, and *mad1*. This study focuses on the bub1-A78V mutant, which is mutated in the Mad3-like region (Taylor *et al.*, 1998; Hardwick *et al.*, 2000; Warren *et al.*, 2002), a domain that is evolutionarily conserved but for which the function is unknown.

MATERIALS AND METHODS

Yeast Methods

The strains used in this study are listed in Table 1. Standard yeast methods and media were used (Moreno *et al.*, 1991). Spotting experiments were performed by growing cells to midlog phase in yeast extract medium (YE) or supplemented Edinburgh minimal media (EMM) and spotting an equal number of cells in fivefold dilutions onto YE or EMM plates in the presence or absence of 15 μg/ml thiabendazole (TBZ, Sigma-Aldrich, 3 mg/ml stock in isopropanol) as indicated. Asynchronous cultures were grown in YE or supplemented EMM to midlog phase at 25°C, whereas SAC-activated cells were grown to midlog phase in YE at 25°C, synchronized in hydroxyurea (HU, 10 mM) for 4 h at 30°C, washed, and released into YE + 25 μg/ml methyl benzimidazol-2yl carbamate (carbendazim, MBC, Sigma-Aldrich, 5 mg/ml stock in dimethyl sulfoxide) for 2 h at 25°C as previously described (Millband and Hardwick, 2002).

Protein Methods

Yeast were grown in YE to midlog phase, and extracts were prepared in HB buffer (Moreno *et al.*, 1991) or trichloroacetic acid (TCA) extraction buffer. For TCA extractions, 8×10^7 cells were washed in 20% TCA, pelleted, resuspended in 100 μL 20% TCA, lysed by bead beating, pelleted, washed with ice-cold acetone, resuspended in 50 μL 2× SDS loading buffer plus 25 μL 1 M Tris (pH 7.5), boiled 5 min, and centrifuged 5 min at 13,000 rpm to collect the supernatant. Proteins were separated by 4–20% or 10% Tris-HCl SDS-PAGE (Bio-Rad, Richmond, CA) and transferred to an Immobilon-P nylon membrane (Millipore, Bedford, MA). A polyclonal rabbit anti-*S. pombe* Bub1p

Table 1. *Schizosaccharomyces pombe* strains used in this study

Strain name	Genotype	Origin
SS446	Wild type *leu1-32 ura4-D18 ade6-M210 h-*	K. Gould
SS447	Wild type *leu1-32 ura4-D18 ade6-M216 h+*	K. Gould
SS104	Wild type *leu1-32 ade6-M216 h+*	P. Nurse
SS45	Wild type *ade6-M210 h+*	P. Nurse
SS1094	*mad1Δ::ura4*$^{+}$ *leu1-32 ura4-D18 h-*	T. Matsumoto
SS638	*mad2Δ::ura4*$^{+}$ *leu1-32 ura4-D18 ade6-M216 h+*	S. Sazer
SS896	*mad3Δ::ura4*$^{+}$ *leu1-32 ura4-D18 ade6-M210 h-*	K. Hardwick
SS894	*bub1Δ::ura4*$^{+}$ *leu1-32 ura4-D18 ade6-M216 h-*	K. Hardwick
SS895	*bub3Δ::ura4*$^{+}$ *leu1-32 ura4-D18 ade6-M210 h+*	K. Hardwick
SS1563	*bub1-A78V leu1-32 ura4-D18 ade6-M216 his3-D1 h-*	This study
SS1564	*bub1-A78V leu1-32 ade6-M216 h+*	This study
SS1565	*bub1-G139D leu1-32 ade6-M210 h-*	This study
SS1566	*bub1-R988Stop leu1-32 ade6-M216 h-*	This study
SS1567	*bub3-G255D leu1-32 ade6-M216 h+*	This study
SS1568	*mad1-Q386Stop leu1-32 ade6-M210 h-*	This study
SS923	Atb2-GFP*::lys1*$^{+}$ *leu1-32 ade6-M210 his3-D1 h-*	R. McIntosh
SS1569	Atb2-GFP*::lys1*$^{+}$ *leu1-32 ade6-M210 his3-D1 pREP41 × -mph1 h-*	This study
SS1570	*bub1-A78V* Atb2-GFP*::lys1*$^{+}$ *leu1-32 ade6-M216 his3-D1 h-*	This study
SS1276	Bub1-GFP*::his3*$^{+}$ *leu1-32 ura4-D18 ade6- his3- h+*	K. Hardwick
SS1571	Bub1-A78V-GFP*::his3*$^{+}$ *leu1-32 ura4-D18 ade6-M216 his3-D1 h-*	This study
SS1277	Bub3-GFP*::his3*$^{+}$ *leu1-32 ura4-D18 ade6- his3- h+*	K. Hardwick
SS1572	Bub3-GFP*::his3*$^{+}$ *bub1-A78V leu1-32 ura4-D18 ade6-M216 h+*	This study
SS1573	Bub3-GFP*::his3*$^{+}$ *bub1Δ::ura4*$^{+}$ *leu1-32 ade6-M216 h+*	This study
SS1278	Mad1-GFP*::his3*$^{+}$ *leu1-32 ura4-D18 ade6-M210 his3- h+*	K. Hardwick
SS1574	mad1-GFP*::his3*$^{+}$ *bub1-A78V leu1-32 ura4-D18 ade6-M216 h+*	This study
SS1575	mad1-GFP*::his3*$^{+}$ *bub1Δ::ura4*$^{+}$ *leu1-32 ade6-M216 h-*	This study
SS1279	Mad3-GFP*::his3*$^{+}$ *leu1-32 ura4-D18 ade6-M210 his3- h+*	K. Hardwick
SS1576	Mad3-GFP*::his3*$^{+}$ *bub1-A78V leu1-32 ura4-D18 ade6-M216 h+*	This study
SS1577	Mad3-GFP*::his3*$^{+}$ *bub1Δ::ura4*$^{+}$ *leu1-32 ura4-D18 ade6-M216 h-*	This study
SS1011	*bub1Δ::leu2*$^{+}$ *leu1-32 ura4-DS/E ade6-M210 his1–102 h+*	J. P. Javerzat
SS1578	*bub1Δ::leu2*$^{+}$ *leu1-32 ura4-DS/E ade6-M210 his1–102 h+* pUR18	This study
SS1579	*bub1Δ::leu2*$^{+}$ *leu1-32 ura4-DS/E ade6-M210 his1–102 h+* pUR19-*bub1*	This study
SS1580	*bub1Δ::leu2*$^{+}$ *leu1-32 ura4-DS/E ade6-M210 his1–102 h+* pUR19-*bub1-A78V*	This study
SS1581	*bub1Δ::ura4*$^{+}$ *ura4-D18 ade6-M216 h-*	This study
SS1582	*bub1Δ::leu2*$^{+}$ *leu1-32 ura4-DS/E ade6-M210 his1–102 h+ int::*pUR19-*bub1-A78V*	This study
SS1583	*bub1Δ::leu2*$^{+}$ *leu1-32 ura4-DS/E ade6-M210 his1–102 h+ int::*pUR19-*bub1*	This study
SS1584	Bub1-GFP*::his3*$^{+}$ *leu1–32 ade6- his3-*	This study
SS1585	Bub1-A78V-SV40NLS-GFP*::his3*$^{+}$ *leu1–32 ade6-M216 his3-D1 h-*	This study
SS1711	Bub1-A78V-nucNLS-GFP*::his3*$^{+}$ *leu1-32 ade6-M216 his3-D1 h-*	This study
SS1712	*leu1-32 ura4-D18 ade6-M210 h-* pREP3X-*GFP*	This study
SS1713	*leu1-32 ura4-D18 ade6-M210 h-* pREP4-*SV40NLS-GFP*	This study
SS1714	*leu1-32 ura4-D18 ade6-M210 h-* pSGP573-*Bub1p Mad3-like domain-GFP*	This study

antibody (K. Hardwick, unpublished results) at 1:1000 dilution in BLOTTO, a 1:5000 dilution of anti-rabbit HRP-conjugated secondary antibody (Promega, Madison, WI), and ECL detection reagents (Amersham, Piscataway, NJ) were used to perform Western blots.

Coimmunoprecipitations were performed essentially as described (Millband and Hardwick, 2002). A polyclonal Bub1p antibody was used to immunoprecipitate wild-type Bub1p and Bub1p-A78V from cycling cells. Coupled 9E10-Sepharose (Santa Cruz Biotechnology, Santa Cruz, CA) was used to immunoprecipitate Myc-Bub3p (K. Hardwick, unpublished results) from wild-type and bub1-A78V cells. Western blots were performed using sheep anti-Bub1p (1:1000 in BLOTTO) and rabbit polyclonal anti-Myc A14 (Santa Cruz Biotechnology, 1:2000 in BLOTTO).

The mph1 *Overexpression Suppressor Screen*

SS446 and SS447 were transformed with pREP41X-*mph1* (He *et al.*, 1998), in which *mph1* is transcribed from the thiamine-repressible *nmt1* gene promoter (Basi *et al.*, 1993; Maundrell, 1993). Transformants were grown in EMM with thiamine to repress *mph1* expression and mutagenized with nitrosoguanidine in two batches to 61.5 or 73.7% killing efficiency. From 1.6×10^7 cells plated onto EMM with appropriate amino acid supplements in the absence of thiamine to induce *mph1* overexpression, four thousand *mph1* overexpression suppressor strains were selected by their growth after replica plating to fresh EMM plates lacking thiamine. Two hundred fifty suppressor strains were streaked to EMM with thiamine + 15 μg/ml TBZ at 29°C to test whether they were likely to be defective in the SAC.

Genetic linkage between *mph1* overexpression suppressor mutations and mutations in the SAC genes, *mad1, mad2, mad3, bub1,* and *bub3,* was determined by random spore analysis. For strains that carry mutations that were tightly linked to one of these checkpoint genes, PCR amplification (Qiagen *Taq*PCR Master Mix Kit, Chatsworth, CA) and automated nucleotide sequencing of the candidate mutated ORF were performed.

Sequence Analysis

S. pombe Bub1p conserved regions were identified based on homology to defined *S. cerevisiae* Bub1p domains (Taylor *et al.*, 1998; Hardwick *et al.*, 2000; Warren *et al.*, 2002) using the European Molecular Biology Open Software Suite water program (http://www.hgmp.mrc.ac.uk/Software/EMBOSS/Apps/water.html). A list of *S. pombe* proteins that contain a putative nuclear localization signal (http://cubic.bioc.columbia.edu/cgi/var/nair/predictNLS/Genome.pl) constructed by the Rost group at the Columbia University Bioinformatics Center (Nair *et al.*, 2003) was used to determine if fission yeast spindle checkpoint proteins contain an identifiable NLS.

Strain Construction

Using a previously described method (Millband and Hardwick, 2002), a plasmid encoding the carboxy terminus of Bub1 fused to GFP (Hardwick, unpublished results) was used to construct Bub1-A78V-GFP by PshAI digestion of the tagging vector and subsequent integration into bub1-A78V cells. To construct bub1-A78V strains carrying Mad1-GFP, Bub3-GFP, Mad3-GFP (Millband and Hardwick, 2002), or Atb2-GFP (Ding *et al.*, 1998), TBZ-sensitive progeny were selected by replica plating random spores to plates containing 15 μg/ml TBZ and phloxine B and subsequently screened visually for the presence of GFP. To construct bub1Δ strains carrying Mad1-GFP, Bub3-GFP, or Mad3-GFP, ura+ colonies were selected from a cross and screened visually for the presence of GFP. To construct Bub1-NLS-GFP and Bub1-A78V-NLS-GFP strains, complementary oligonucleotides (Integrated DNA Technologies, Coralville, IA) encoding the SV40 or nucleoplasmin NLS (for review see Yoshida and Sazer, 2004) were hybridized by boiling for 5 min and slowly cooling to room temperature and cloned into the *Sal*I site of the Bub1-GFP tagging vector, located between the *bub1* C terminus and *GFP*. The cloned products were integrated into wild-type or bub1-A78V cells as described above. To construct a GFP-tagged Bub1p Mad3-like domain (amino acids 6–193), this region was PCR amplified and cloned into the *Bgl*II site of pSGP573 (Pasion and Forsburg, 1999), and the resulting plasmid was transformed into wild-type cells. To construct pREP4-SV40NLS-GFP, a fragment from pREP SV40 NLS-GFP-LacZ (Yoshida and Sazer, 2004) containing only SV40 NLS-GFP was subcloned into pREP4 (Maundrell *et al.*, 1993).

Site-directed Mutagenesis

To recreate the bub1-A78V mutation, a wild-type *bub1* genomic plasmid (Bernard *et al.*, 1998) was mutagenized using the Transformer Site-directed Mutagenesis Kit (Clontech, Palo Alto, CA). The *bub1-A78V* mutagenic oligonucleotide was: 5′-ggtcaacaaaatgcttgatgatgttattcagtacttagaaagatg-3′.

Fluorescence Microscopy

To observe microtubules in living cells, Atb2-GFP cells (Ding *et al.*, 1998) expressing GFP-α-tubulin were grown in supplemented EMM in the presence of thiamine at 25°C, washed, and resuspended in supplemented EMM lacking thiamine for 24 h at 32°C. Strains expressing untagged GFP, SV40NLS-GFP, or Bub1p Mad3-like-domain-GFP were grown in the presence of thiamine at 25°C, washed, and resuspended in EMM lacking thiamine for 18 or more hours at 25°C. These four strains were visualized using a Zeiss Axioskop fluorescence microscope (Thornwood, NY) and photographed with a DVC 1300 Black and White CCD camera using QED software (Media Cybernetics, Silver Spring, MD). Bub1-GFP, Bub1-A78V-GFP, Bub1-A78V-SV40NLS-GFP, Bub1-A78V-nucNLS-GFP, Bub3-GFP, Mad1-GFP, or Mad3-GFP were visualized using an Applied Precision DeltaVision Restoration Microscope System (Issaquah, WA), and images were acquired using a Photometrics CoolSnap HQ camera (Tucson, AZ). Equivalent exposure times and equal numbers of 0.25-μm stacks were used to accumulate a series of optical images that were summarized as two-dimensional projections. To calculate the ratio of nuclear GFP signal to cytoplasmic GFP signal, fluorescence values were determined using the sum algorithm of the DeltaVision software (SoftWoRx 3.2.3) to analyze stacks of Z sections, which were projected two-dimensionally using the sum protocol. Fluorescence values were determined for the nucleus and cytoplasm of at least five cells per sample, and the statistical SE was calculated for each data set. Hoechst 33342 (Sigma-Aldrich, St. Louis, MO) or 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich) were used to visualize DNA in living or fixed cells respectively, as previously described (Moreno *et al.*, 1991; Demeter *et al.*, 1995). To determine the percentage of cells with condensed DNA, cells were fixed with formaldehyde, stained with DAPI, and visualized using a Zeiss Axioskop fluorescence microscope.

RESULTS

The **mph1** *Overexpression Screen Identified Five New Alleles of Known SAC Genes*

The observation that *S. pombe* cells mutated in *mad2* are insensitive to the checkpoint activation and cell cycle arrest caused by *mph1* overexpression (He *et al.*, 1998) suggested that this characteristic could be used to identify strains mutated in other checkpoint genes that act downstream of *mph1*.

Approximately 1.6×10^7 cells, transformed with a plasmid from which *mph1* was expressed from the thiamine-repressible *nmt1* gene promoter, were grown in thiamine to keep the level of expression low and then chemically mutagenized (see *Materials and Methods* for details). Four thousand candidate suppressor strains were identified by their ability to form colonies when expression of *mph1* was derepressed. To ask whether this screening strategy identified strains with defects in microtubules and/or components of the spindle assembly checkpoint, 250 of the suppressor strains were tested for growth in the presence or absence of the microtubule-destabilizing drug, TBZ. From among the 55 most TBZ-sensitive strains, 16 were initially chosen for further study because of their high sensitivity to TBZ and normal growth in the absence of TBZ.

To determine if they were mutated in a previously identified SAC gene, each of the 16 strains was crossed to mad1Δ, mad2Δ, mad3Δ, bub1Δ, and bub3Δ strains (He *et al.*, 1997, 1998; Bernard *et al.*, 1998; Millband and Hardwick, 2002). Five of the strains carried a mutation that is tightly linked to one of these checkpoint genes. PCR amplification of these putative mutant alleles and subsequent nucleotide sequencing of their open reading frames confirmed that three *bub1* alleles, one *bub3* allele, and one *mad1* allele were identified, indicating that the strategy of the screen was valid.

The three *bub1* mutations occurred in evolutionarily conserved regions of the protein (Figure 1A). The *bub1-R988Stop* mutation introduced a premature stop codon in the protein kinase domain and removed 82 amino acids from the carboxy terminus of the protein. The *bub1-A78V* and *bub1-G139D* mutations both introduced a single amino acid change in the conserved Mad3-like region. Strains carrying each of these three mutant alleles exhibited a 25- to 625-fold increase in sensitivity to TBZ when compared with wild-type cells.

The bub1-A78V Mutant Is Defective in the SAC but Produces a Wild-type Level of Bub1p

A Western blot was performed to determine whether the three bub1 mutants are checkpoint-defective because, like bub1Δ, they have a significant decrease in Bub1 protein level. The amounts of Bub1 protein in the bub1-R988Stop and bub1-G139D strains were significantly lower than in the wild-type strain, indicating that they are likely to be checkpoint-defective because of the near absence of Bub1p. In contrast, bub1-A78V produces a wild-type level of protein (Figure 2A). To confirm this finding, a Western blot was performed using serial dilutions of the same protein preparations shown in Figure 2A, and the band intensities were quantified (Figure 2B). For the wild-type sample, the ratio of Bub1p band intensity to the intensity of the control band was 3.1. This ratio was 3.7 for the bub1-A78V sample, demonstrating that the ability of bub1-A78V cells to survive *mph1* overexpression was not due to a decreased abundance of Bub1 protein.

The only mutation identified by sequencing the *bub1-A78V* allele (C233T) resulted in an alanine to valine mutation at amino acid position 78. This alanine is not conserved among Bub1 proteins but is located within a highly conserved region of the protein. To confirm that the single amino acid change in Bub1p-A78V is sufficient to disrupt SAC function, the mutation was recreated by site-directed mutagenesis of a plasmid that was then transformed into bub1Δ cells to determine whether it encodes a defective protein. Although wild-type *bub1* expressed from its native promoter in a multicopy plasmid rescued the TBZ-sensitivity of the bub1Δ strain, the bub1-A78V plasmid only marginally improved growth (Figure 2C). Similar results were observed when the plasmids were integrated into bub1Δ cells (Figure 2D) and are consistent with the previous comparison between bub1-A78V and bub1Δ strains on TBZ plates (Figure 1B), suggesting that the mutant protein may retain partial function. These results indicate that the bub1-A78V mutation is solely responsible for the defects in the bub1-A78V strain.

Therefore, the bub1-A78V mutant strain can be used to examine the functional consequences of specifically disrupting the Bub1p Mad3-like region. Compared with wild-type cells, the growth of bub1-A78V cells was impaired when the SAC was activated by TBZ (Figure 1B), indicating that bub1-A78V cells fail to activate the SAC. To confirm that the SAC is not functional in bub1-A78V cells, it was activated in the

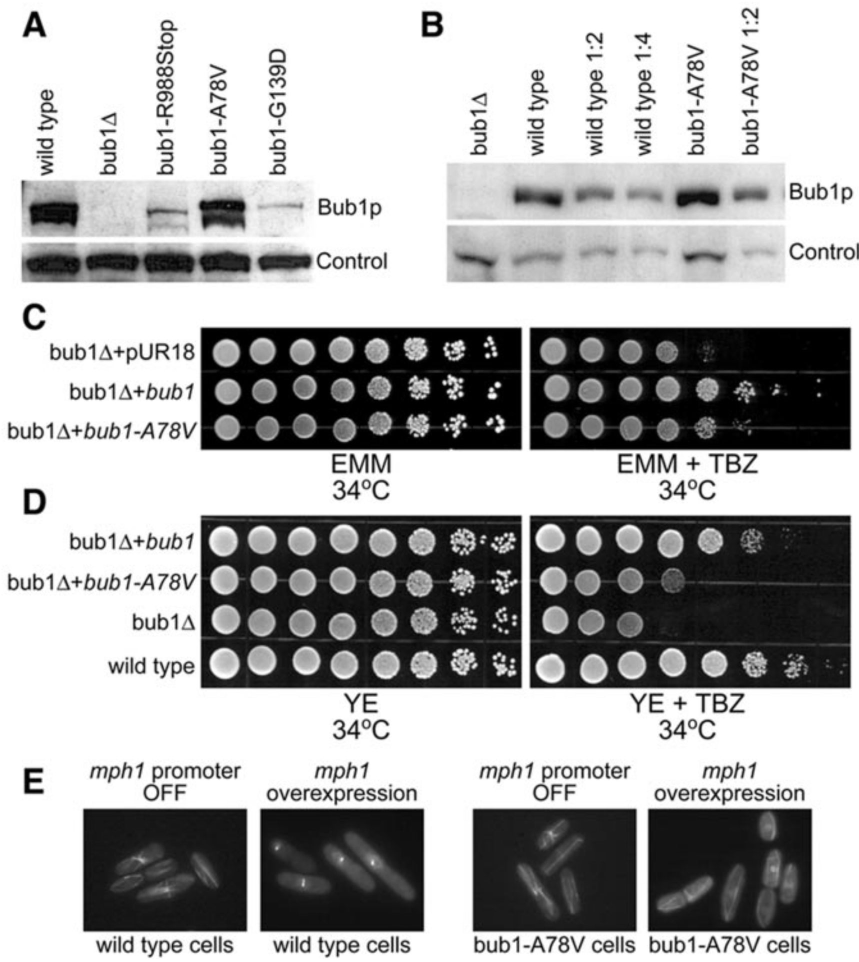


Figure 2. The bub1-A78V strain produces stable Bub1p but is sensitive to TBZ and cannot arrest the cell cycle in response to *mph1* overexpression. (A and B) Western blots using an anti-Bub1p polyclonal antibody to compare Bub1p levels in the bub1 point mutants to wild-type and bub1Δ cells. The bub1-A78V strain, but not the bub1-R988Stop or bub1-G139D strains, produces the wild-type level of Bub1p. (B) Serial dilutions of the wild-type and bub1-A78V protein preparations shown in A. (C) Site-directed mutagenesis was used to recreate *bub1-A78V* on a plasmid. bub1Δ cells were transformed with empty vector (pUR18), wild-type *bub1*, or *bub1-A78V*. Cells were grown to midlog phase in minimal media to select for the plasmid and spotted in fivefold dilutions onto plates in the presence or absence of TBZ. (D) Plasmids containing wild-type *bub1* or *bub1-A78V* were integrated into bub1Δ cells at the endogenous *bub1* locus behind the *bub1* promoter. Strains were grown to midlog phase in rich medium and spotted in fivefold dilutions on YE plates. (E) *mph1* was overexpressed in wild-type and bub1-A78V cells expressing GFP-tubulin to visualize microtubules. The presence of short mitotic spindles in wild-type cells overexpressing *mph1* indicates an arrest at the metaphase to anaphase transition. bub1-A78V cells with short spindles do not accumulate under these conditions.

absence of microtubule disruption by *mph1* overexpression. Under these conditions, 18.5% of wild-type cells arrested at the metaphase-to-anaphase transition (Figure 2E) with a short mitotic spindle. In contrast, only 1.5% of bub1-A78V cells exhibited a short spindle after this treatment. This result confirmed that bub1-A78V cells are SAC-defective because they fail to arrest the cell cycle in response to SAC activation, which explains their ability to survive *mph1* overexpression, during which the microtubules are unperturbed, and their impaired growth in the presence of TBZ. Taken together, these data demonstrate that alanine 78 in the Bub1p Mad3-like region is required for SAC activation in response to *mph1* overexpression or microtubule damage.

The Bub1p, Mad3p, and Bub3p SAC Proteins, but not Mad1p, are Mis-localized in Interphase bub1-A78V Cells

Wild-type *S. pombe* Bub1-GFP localized predominantly but not exclusively to the nucleus in interphase cells (Figure 3B), although it does not contain a consensus nuclear localization signal (see *Materials and Methods*). To further characterize the checkpoint defect caused by the bub1-A78V mutation, a strain that expresses *bub1-A78V-GFP* from the endogenous promoter at its genomic locus was constructed. A Western blot showed that Bub1-A78V-GFP was produced at the same level as wild-type Bub1-GFP and had the predicted molecular mass of 145 kDa (Figure 3A). Although Bub1-GFP accumulated predominantly inside of the nucleus in cycling cells, the nuclear accumulation of Bub1-A78V-GFP was significantly reduced (Figure 3B). The ratio of nuclear to cytoplasmic Bub1-GFP signal was 7.4 ± 0.7 in wild-type cells, but only 1.2 ± 0.1 in Bub1-A78V-GFP cells (Figure 3F). A Western blot confirmed that the observed decrease in nuclear localized Bub1-A78V-GFP was not reflective of a lower level of Bub1-A78V-GFP compared with Bub1-GFP (Figure 3A). These data demonstrate that disrupting the Mad3-like domain of Bub1p alters its intracellular localization during interphase.

Bub1p binds to Bub3p in fission yeast (Hardwick, unpublished results), budding yeast, and higher eukaryotes, and Bub3p constitutively binds Mad3p (for review see Millband and Hardwick, 2002). In addition, Bub1p, Bub3p, and Mad1p form a complex in budding yeast (Brady and Hardwick, 2000). Therefore the localization of the Mad1, Bub3, and Mad3 checkpoint proteins was monitored in cycling bub1-A78V cells. In wild-type cells, Mad1p-GFP localized to the nuclear envelope in a punctate pattern (Figure 3C) that is indicative of Mad1p's possible association with nuclear pore complexes, as reported in budding yeast, *Xenopus* oocytes, and human cells (Chen *et al.*, 1998; Campbell *et al.*, 2001; Iouk *et al.*, 2002). This localization was unaltered in bub1-A78V or bub1Δ cells, indicating that Bub1p is not required for Mad1p nuclear localization in *S. pombe*. Bub3-GFP and Mad3-GFP were more concentrated in the nucleus compared with the cytoplasm in wild-type cells (Figure 3, D and E), but this nuclear accumulation was diminished in bub1-A78V cells. This pattern of mis-localization was similar to that observed in the bub1Δ strain (Figure 3, D and E).

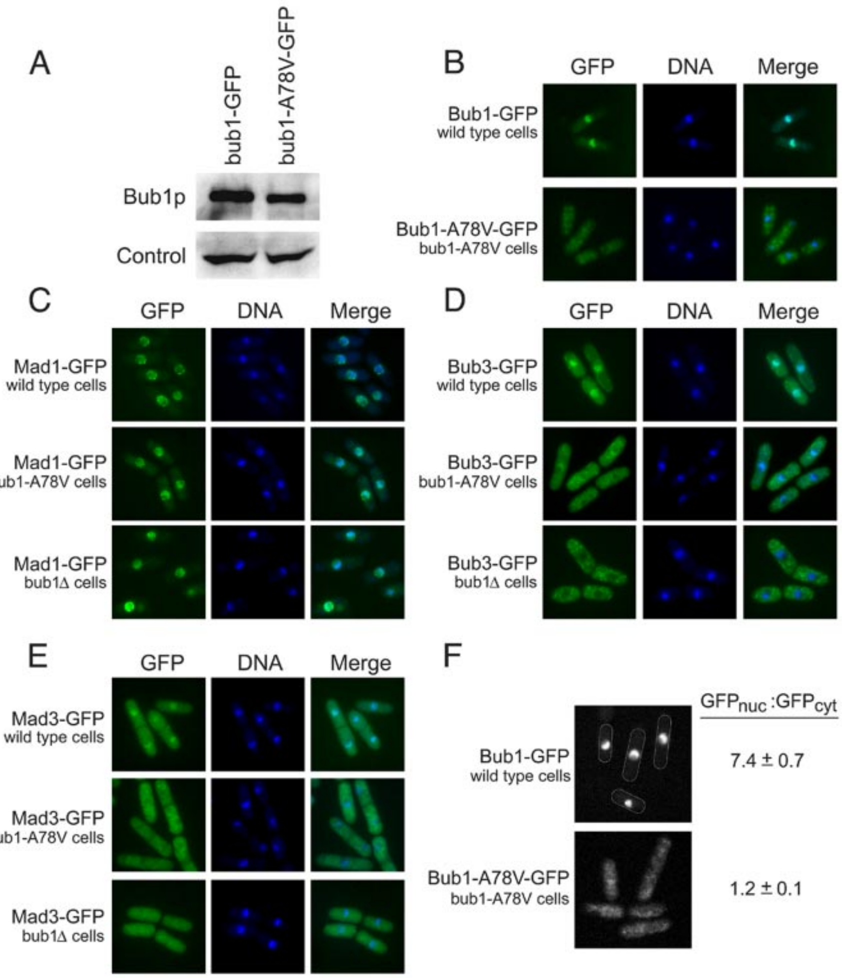


Figure 3. Bub1-GFP, Bub3-GFP, and Mad3-GFP are mis-localized in the bub1-A78V mutant. (A) Western blot using an anti-Bub1p polyclonal antibody to demonstrate production of Bub1-A78V-GFP. (B) Strains expressing C terminally GFP-tagged *bub1* or *bub1-A78V* driven by the native *bub1* promoter at its endogenous locus were cultured in rich medium (YE). Live cells were examined using an Applied Precision DeltaVision microscope to observe localization of the GFP-tagged protein, and DNA was detected by staining with Hoechst 33342. (C–E) Strains expressing C terminally GFP-tagged *Mad1* (C), *Bub3* (D), or *Mad3* (E) driven by their native promoters at their endogenous loci were cultured and observed as in B. (C) Wild-type, bub1-A78V, and bub1Δ cells expressing Mad1-GFP. (D) Wild-type, bub1-A78V, and bub1Δ cells expressing Bub3-GFP. (E) Wild-type, bub1-A78V, and bub1Δ cells expressing Mad3-GFP. (F) Bub1-GFP and Bub1-A78V-GFP cells were grown in supplemented EMM and examined as above. The nuclear to cytoplasmic GFP ratio was calculated by obtaining nuclear and cytoplasmic fluorescence intensity values using SoftWoRx 3.2.3 software.

These results indicate that in bub1-A78V cells, Bub1p, Bub3p, and Mad3p, but not Mad1p, fail to correctly accumulate in the nucleus of cycling cells.

Bub1-A78V-GFP Does Not Localize to Kinetochores during a Normal Mitosis or When the SAC Is Activated

During a normal fission yeast cell cycle, Bub1p localizes to two spots, corresponding to clustered kinetochores, during prometaphase and remains there until telophase (Bernard *et al.*, 1998). When the SAC is activated, Bub1p is recruited to kinetochores, where it remains until the cell cycle resumes (Bernard *et al.*, 1998; Toyoda *et al.*, 2002). To test whether the bub1-A78V mutant is defective in the SAC because Bub1p cannot be properly recruited to kinetochores, Bub1-GFP and Bub1-A78V-GFP localization was compared in cycling interphase cells and in cells synchronized in mitosis upon release from an S phase block imposed by HU into either fresh medium or medium containing the microtubule-destabilizing drug, MBC, which rapidly promotes depolymerization of microtubules in fission yeast (Tran *et al.*, 2001; Sawin and Snaith, 2004) and activates the SAC (Millband and Hardwick, 2002).

In untreated cycling or mitotic cells, Bub1-GFP was predominantly nuclear, whereas Bub1-A78V-GFP was more uniformly distributed between the nucleus and the cytoplasm (Figure 4A). Bub1-GFP localized to kinetochore dots in early and late anaphase mitotic cells (Figure 4A). This distinct localization pattern was not seen with Bub1-A78V-GFP (Figure 4A). When the SAC was activated by MBC, 20.6 ± 1.9% of wild-type cells exhibited bright Bub1-GFP kinetochore dots, a number consistent with previously published reports (Tournier *et al.*, 2004). This localization pattern was only observed in 3.0 ± 0.6% bub1-A78V-GFP cells. These results demonstrate that Bub1p-A78V is defective in kinetochore localization. To ensure that this observed mislocalization did not result because fewer mitotic bub1-A78V cells were present in the sample population due to the SAC defect, cells were examined 2 h after SAC activation. At this time point, equal numbers of mitotic cells were observed in the wild-type and mutant strains (see Figure 4B).

Mad1p Localizes Correctly to Kinetochores when the SAC Is Activated in bub1-A78V and bub1Δ Cells

Mad1p is recruited to unattached kinetochores when the SAC is activated in fission yeast (Hardwick, unpublished results), budding yeast (Gillett *et al.*, 2004), and higher eukaryotes (for review see Bharadwaj and Yu, 2004), and experiments in budding yeast and *Xenopus laevis* show that this recruitment requires Bub1p (Sharp-Baker and Chen, 2001; Gillett *et al.*, 2004). In *S. pombe*, Mad1p localization to the nucleus and enrichment at the nuclear periphery in interphase was the same in wild-type, bub1-A78V, and bub1Δ cells (Figures 3C and 5A). When the checkpoint was activated by MBC treatment for 2 h, Mad1-GFP localized to kinetochores in wild-type, bub1-A78V, and bub1Δ cells (Figure 5A). These results indicate that Bub1p is

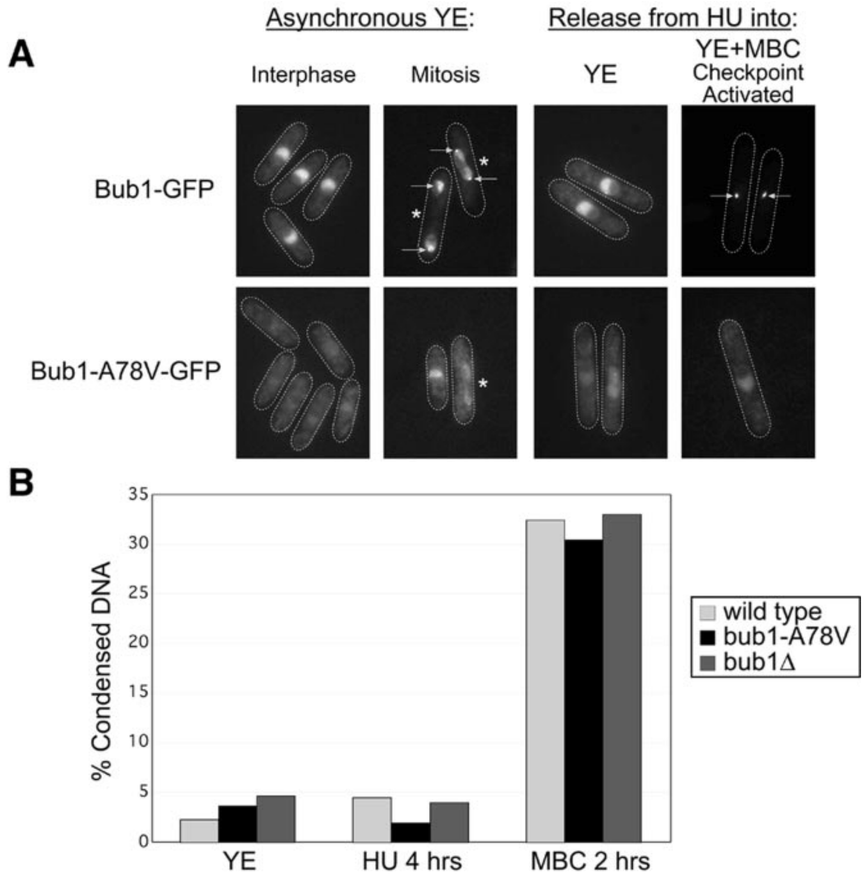


Figure 4. Bub1-A78V-GFP does not localize correctly to the nucleus during interphase or to kinetochores during mitosis or checkpoint activation. (A) Asynchronous cells expressing C terminally tagged *bub1-GFP* or *bub1-A78V-GFP* driven by the native *bub1* promoter at its genomic locus were cultured in rich YE medium (Asynchronous YE) or synchronized in S phase by hydroxyurea (HU) to enrich for mitotic cells upon release into YE or into YE with MBC (YE+MBC), which activates the SAC. GFP localization was observed in live cells. Kinetochore localization of wild-type Bub1p (arrows) occurs during a normal mitosis (asterisk) or when the SAC is activated by MBC. In mitotic or SAC-activated bub1-A78V cell images, the GFP fluorescence was enhanced to reveal that Bub1-A78V-GFP is not excluded from the nucleus in mitotic or SAC-activated cells. (B) Wild-type, bub1-A78V or bub1Δ cells were grown in YE (YE), synchronized in HU for 4 h (HU 4 h), or synchronized in HU and then released into YE+MBC for 2 h (MBC 2 h). Cells were fixed in formaldehyde and stained with DAPI to visualize DNA, and the percentage of cells with condensed DNA was determined.

not required for any aspect of Mad1p intracellular localization. Yet, neither bub1-A78V nor bub1Δ cells arrested at metaphase in response to checkpoint activation, demonstrating that the kinetochore recruitment of Mad1p alone is unable to promote a SAC response.

Bub3p and Mad3p Do Not Localize to Kinetochores when the SAC Is Activated in bub1-A78V or bub1Δ Cells

In *Xenopus* extracts and *Drosophila* cells, Bub3p localization to unattached kinetochores requires Bub1p (Basu *et al.*, 1998; Sharp-Baker and Chen, 2001), and experiments in *S. pombe* have shown that Mad3p localization to unattached kinetochores is dependent on Bub1p (Millband and Hardwick, 2002). The observation that the interphase nuclear accumulation of Bub3p and Mad3p was reduced in bub1-A78V and bub1Δ cells (Figure 3, D and E) raised the question of whether Bub3-GFP and Mad3-GFP can localize to kinetochores when the SAC is activated in bub1-A78V cells.

In wild-type cycling cells or synchronized mitotic cells, Bub3-GFP and Mad3-GFP localized predominantly inside the nucleus, but this nuclear accumulation was reduced in bub1-A78V and bub1Δ cells (Figure 5, B and C). When the checkpoint was activated by MBC treatment for 2 h, Bub3-GFP and Mad3-GFP localized to kinetochores in wild-type cells but not in bub1-A78V or bub1Δ cells (Figure 5, B and C). This mis-localization of Bub3-GFP and Mad3-GFP was not a result of decreased production of Bub1p-A78V in these cells (Figure 5D).

Bub1p-A78V Retains Its Ability To Bind to Bub3p

Because Bub1p binds to Bub3p constitutively in budding yeast and higher eukaryotes, and Bub3p is required for kinetochore localization of Bub1p (for review see Millband *et al.*, 2002), it is possible that Bub1p-A78V does not localize correctly because mutation of the Mad3-like region prevents its binding to Bub3p. This question was addressed by asking if these two proteins coimmunoprecipitate. When Bub1p or Bub1p-A78V was immunoprecipitated from cycling cells, Bub3p was copurified (Figure 6). Likewise, when Bub3p was immunoprecipitated from cycling cells, Bub1p or Bub1p-A78V was coprecipitated. Therefore, the SAC defect in bub1-A78V cells is not a result of disruption of the Bub1p-Bub3p complex.

Increased Nuclear Accumulation of Bub1p-A78V Does Not Significantly Improve Kinetochore Targeting of Bub1p-A78V or SAC Function

Bub1p-A78V did not localize to kinetochores during SAC activation and failed to accumulate to the wild-type level in the nucleus. The SAC defect caused by the A78V mutation may result from either or both of these defects, because adequate nuclear accumulation in interphase could be required for sufficient kinetochore localization upon SAC activation. To distinguish between these possibilities, an exogenous SV40 or nucleoplasmin nuclear localization signal (NLS), each of which functions efficiently in *S. pombe* (for review see Yoshida and Sazer, 2004), was fused to Bub1p-A78V in an attempt to drive more protein into the nucleus.

bub1p-A78V-SV40NLS-GFP or *bub1p-A78V-nucNLS-GFP* was expressed from the genomic *bub1* promoter at the endogenous locus. Although the ratio of nuclear to cytoplasmic GFP fluorescence in cells expressing Bub1-A78V-GFP was 1.1 ± 0.1, this ratio was increased to 2.9 ± 0.2 in

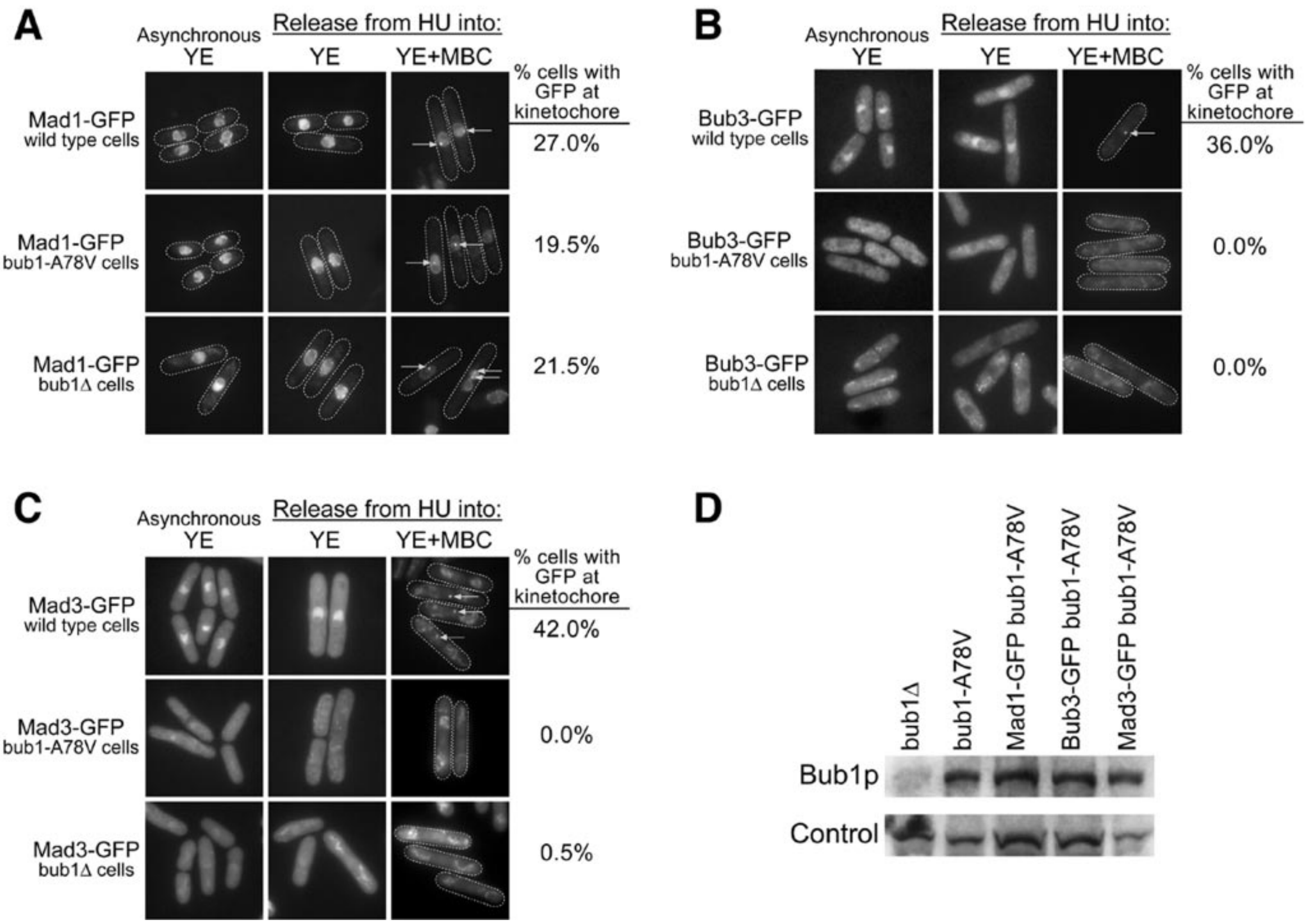


Figure 5. Bub3-GFP and Mad3-GFP do not localize to kinetochores when the SAC is activated in bub1-A78V cells. Asynchronous wild-type, bub1-A78V, and bub1Δ cells expressing C terminally GFP-tagged *Mad1*, *Bub3*, or *Mad3* driven by their native promoters at their endogenous loci were cultured in rich YE medium (Asynchronous YE) or synchronized in S phase by hydroxyurea (HU) to enrich for mitotic cells upon release into YE (YE) or into YE with MBC (YE+MBC), which activates the SAC. GFP localization was observed in live cells. Arrows denote kinetochore localization of the GFP-tagged protein. The percentage of cells exhibiting kinetochore localization of the GFP fusion protein was determined 2 h after release into YE+MBC. (A) Wild-type, bub1-A78V and bub1Δ cells expressing Mad1-GFP. (B) Wild-type, bub1-A78V, and bub1Δ cells expressing Bub3-GFP. (C) Wild-type, bub1-A78V, and bub1Δ cells expressing Mad3-GFP. (D) Western blot using an anti-Bub1p polyclonal antibody to determine levels of Bub1p-A78V in the strains examined in A–C.

bub1-A78V-SV40NLS-GFP cells and 2.0 ± 0.1 in bub1-A78V-nucNLS-GFP cells (Figure 7A). However, the increased nuclear accumulation of Bub1p-A78V did not significantly improve growth on TBZ (Figure 7B). Although a slight improvement in kinetochore targeting was observed when either exogenous NLS was fused to Bub1p-A78V (Figure 7C), these observed differences were not statistically significant as determined by an unpaired *t* test. These data indicate that the bub1-A78V mutation impairs SAC activity by disrupting two aspects of Bub1p function, namely nuclear accumulation and kinetochore localization. Because the kinetochore localization defect could not be overcome by increased nuclear accumulation of the mutant protein, these results indicate that the kinetochore localization defect is not a direct consequence of the nuclear accumulation defect.

The Bub1p Mad3-like Region has NLS Activity

S. pombe Bub1p-GFP is localized predominantly to the nucleus (Figure 3B) although it does not contain a consensus nuclear localization signal (see *Materials and Methods*). However, the A78V mutation in the Mad3-like domain disrupts the nuclear accumulation of Bub1p. To determine if this domain is sufficient to promote nuclear accumulation, the Mad3-like region (amino acids 6–193) was fused to GFP, and the localization of this fusion protein was monitored in wild-type cells. When untagged GFP was expressed in wild-type cells, the GFP fluorescence was distributed throughout the cell (Figure 8A), whereas robust nuclear fluorescence was observed when the classical SV40 NLS was fused to GFP (Figure 8B). Fusing the Bub1p Mad3-like region to GFP also promoted nuclear accumulation, although to a lower level than the SV40 NLS. These results indicate that the Bub1p Mad3-like region is sufficient to direct an exogenous protein into the nucleus.

DISCUSSION

The mph1 *Overexpression Survival Screen Identified Point Mutations in* bub1, bub3, *and* mad3

A screen to identify fission yeast mutants that survive an *mph1* overexpression-induced SAC arrest identified novel mutant alleles of *bub1*, *bub3,* and *mad3*. The *bub1-A78V* mutation is in the conserved Mad3-like domain (Taylor *et al.*,

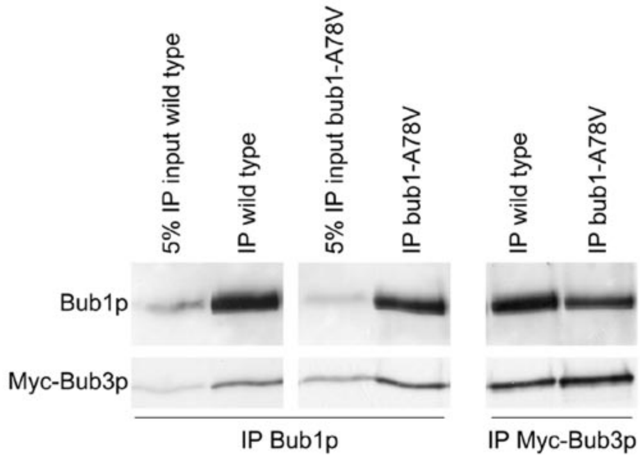


Figure 6. Bub1p-A78V can bind to Bub3p. Wild-type Bub1p or Bub1p-A78V was immunoprecipitated from cells expressing Myc-Bub3p using a polyclonal antibody generated against Bub1p. Half of the immunoprecipitated wild-type Bub1p was loaded onto the gel for comparison with the immunoprecipitated Bub1p-A78V. To immunoprecipitate Myc-Bub3p from wild-type or bub1-A78V cells, coupled 9E10-Sepharose was used. Western blots were probed with a sheep anti-Bub1p antibody to detect Bub1p and a rabbit polyclonal anti-Myc antibody to detect Myc-Bub3p.

1998; Hardwick *et al.,* 2000; Warren *et al.,* 2002), the functional significance of which was previously unknown. Because it produces a stable protein, *bub1-A78V* provides a unique tool for investigating the importance of the Mad3-like region for checkpoint function. Cells in which the Mad3-like domain was deleted from Bub1p (Hardwick, unpublished results) have defects in SAC function and kinetochore targeting similar to those described here for the bub1-A78V point mutant, strengthening our hypothesis that this mutation disrupts the function of the Mad3-like domain.

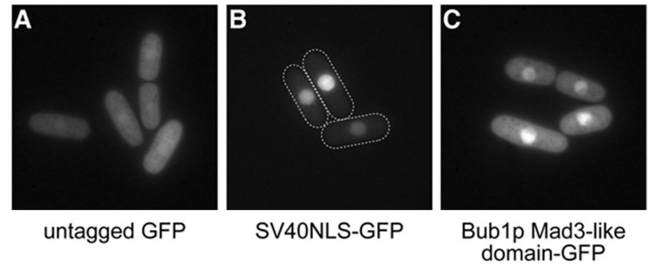
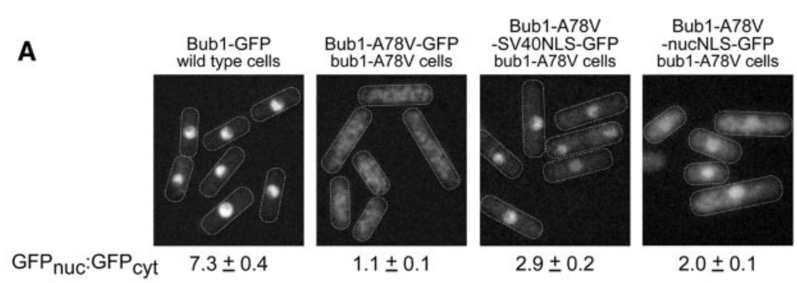


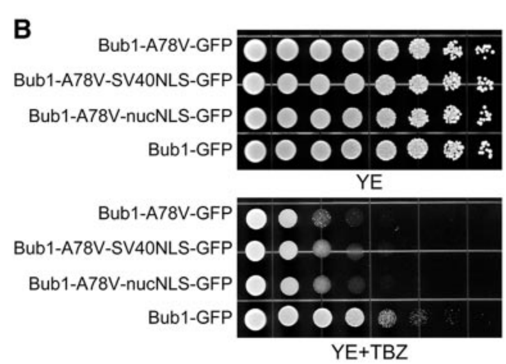
Figure 8. The Bub1p Mad3-like region has NLS activity. Untagged GFP (A), GFP fused to the SV40 NLS (B), or GFP fused to the Bub1p Mad3-like region (amino acids 6–193; C) were expressed in wild-type cells from the thiamine-repressible *nmt1* promoter in a multicopy plasmid. Cells were grown in supplemented EMM lacking thiamine for at least 18 h to induce expression. GFP localization was visualized in live cells.

Disruption of the Mad3-like Domain Interferes with the Nuclear Accumulation and Kinetochore Targeting of Bub1p

Because the nuclear envelope remains intact throughout the cell cycle in yeast, all proteins necessary for nuclear functions, including SAC proteins, must be transported into the nucleus. Bub1p, but not Bub1p-A78V, accumulates in the nucleus of interphase fission yeast cells and is enriched at kinetochores during normal mitoses and upon checkpoint activation (Figure 4A). Even when the GFP fluorescence was enhanced to demonstrate that Bub1p-A78V is not excluded from the nucleus, kinetochore localization was not observed in mitotic cells. Increasing the intranuclear concentration of Bub1p-A78V by fusing it to two different exogenous NLSs did not restore kinetochore localization or SAC function (Figure 7). The Bub1p-A78V mutant protein is therefore defective in kinetochore targeting.

These data raise the question of whether the Mad3-like domain is both necessary and sufficient for the nuclear and kinetochore accumulation of Bub1p. The observations that





C

Strain	% cells with kinetochore GFP
Bub1-GFP	*20.6 ± 1.9
Bub1-A78V-GFP	* 3.0 ± 0.6^
Bub1-A78V-SV40NLS-GFP	4.6 ± 0.9^
Bub1-A78V-nucNLS-GFP	7.3 ± 1.9^

Figure 7. An exogenous NLS increases nuclear accumulation of Bub1-A78V-GFP but does not improve SAC function or kinetochore targeting. (A) Cells expressing *bub1-GFP*, *bub1-A78V-GFP*, *bub1-A78V-SV40NLS-GFP,* or *bub1-A78V-nucNLS-GFP* from the endogenous promoter at the *bub1* genomic locus were grown in supplemented EMM and visualized on an Applied Precision DeltaVision microscope. The nuclear to cytoplasmic GFP ratio was calculated by obtaining nuclear and cytoplasmic fluorescence intensity values using SoftWoRx 3.2.3 software. (B) Cells were grown to midlog phase in rich medium and spotted in fivefold dilutions onto YE plates or YE plates containing 15 μg/ml TBZ at 34°C. (C) The percentage of cells exhibiting kinetochore localization of the GFP fusion protein was determined during SAC activation by MBC treatment for 2 h after HU synchronization. *Significantly different based on an unpaired two-tailed t test (p = 0.0002). ^ Not statistically different based on an unpaired t test (p = 0.14, 0.11).

Bub1p-A78V does not accumulate in the nucleus and that the isolated Mad3-like domain has NLS activity when fused to GFP (Figure 8) show that this domain is necessary and sufficient to promote nuclear targeting. In contrast, the Mad3-like domain alone is not sufficient for kinetochore targeting (unpublished data).

Bub1p-A78V mis-localization results in the mis-localization of Bub3p and Mad3p, but not Mad1p

Fission yeast Bub1p is required for the kinetochore localization and nuclear accumulation of Bub3p and Mad3p and for checkpoint activation. Conversely, the kinetochore localization of Bub1p may require Bub3p and Mad3p, as has been shown in higher eukaryotes (Taylor *et al.*, 1998; Chen, 2002). If so, the decreased nuclear accumulation of Bub3p and Mad3p in bub1-A78V cells could further reduce the ability of mutant Bub1p to localize to kinetochores.

Bub3p Localization Depends on Bub1p Consistent with previous studies in higher eukaryotes (Basu *et al.*, 1998; Sharp-Baker and Chen, 2001), Bub3p localization to kinetochores depends on functional Bub1p. In cells carrying the *bub1-A78V* mutation, Bub3p also fails to accumulate in the nucleus. In both yeast and higher eukaryotes, Bub1p constitutively binds to Bub3p (for review see Millband *et al.*, 2002), and this interaction is maintained in the bub1-A78V mutant. Therefore, the mis-localization of Bub1p-A78V may directly prevent the Bub3p to which it is bound from localizing properly.

Mad3p Localization Depends on Bub1p Mad3-GFP does not localize to kinetochores when the checkpoint is activated in either bub1Δ cells in fission yeast (Millband and Hardwick, 2002) or in the bub1-A78V mutant (Figure 5C). Although binding of Bub1p to Mad3p has not been reported, the human homologue of Mad3p, BubR1, does physically interact with Bub1p (Taylor *et al.*, 2001). A similar interaction between *S. pombe* Bub1p and Mad3p would provide a possible explanation for the mis-localization of Mad3p in bub1-A78V cells. Alternatively, because Bub3p constitutively binds to Mad3p (for review see Millband *et al.*, 2002), failure of Bub3p to accumulate at kinetochores could mis-localize Mad3p.

Mad1p Localization Does Not Depend on Bub1p In budding yeast and frogs, Bub1p recruits Mad1p to unattached kinetochores (Sharp-Baker and Chen, 2001; Gillett *et al.*, 2004). In contrast, the localization of Mad1p does not depend on Bub1p in fission yeast (Figure 5A), which may be explained by the observation that fission yeast Mad1p, but not its budding yeast homologue, has a consensus NLS. Therefore, a Mad1p-Bub1p-Bub3p complex, which forms during mitosis in *S. cerevisiae* (Brady and Hardwick, 2000), is not required for Mad1p targeting to kinetochores in *S. pombe*. Fission yeast Mad1p may have its own kinetochore-targeting domain or may rely on a protein other than Bub1p, Bub3p, or Mad3p for its localization.

Model for Regulation of the intracellular localization of Bub1p

Nuclear Accumulation May Be Regulated by Nuclear Import The A78V mutation in the Mad3-like domain of Bub1p interferes with its nuclear accumulation, although Bub1p does not contain an identifiable NLS (see *Materials and Methods*; Nair *et al.*, 2003). Bub1p may carry an atypical NLS or it may bind to another protein that mediates its nuclear import. Of the proteins known to bind to Bub1p, neither Bub3p nor Mad3p have an identifiable NLS sequence.

Both the SV40 and the nucleoplasmin NLS can target *S. pombe* proteins exclusively to the nucleus (see Yoshida and Sazer, 2004), yet they are unable to restore the wild-type level of Bub1p-A78V to the nucleus. This observation and the lack of exclusive nuclear localization of wild-type Bub1p are consistent with the hypothesis that Bub1p has both an NLS and a nuclear export signal (NES) and that it shuttles between the nucleus and the cytoplasm. If so, Bub1p's intracellular localization could be regulated by the balance between nuclear import and export and/or by the retention of Bub1p in the nucleus.

Nuclear Accumulation of Bub1p May Be Regulated by Nuclear Retention Bub1p may bind to a nuclear anchoring protein that retains the protein in the nucleus, but such an anchor is unlikely to be Bub3p, because the bub1-A78V mutation does not disrupt the Bub1p-Bub3p complex.

Another candidate for the Bub1p nuclear anchor in interphase cells is the kinetochore, although such an interaction may be too transient to have been observed previously. Consistent with this possibility is a report that Bub1p colocalizes with kinetochore proteins recognized by the CREST antiserum in interphase human cells (Ouyang *et al.*, 1998). If this were the case in *S. pombe*, the inability of Bub1p-A78V to bind to kinetochores in interphase cells might contribute to the defects in both nuclear and kinetochore accumulation in mitotic or checkpoint-activated cells.

SUMMARY

The kinetochore localization of Bub1p and its checkpoint function are abrogated by the A78V point mutation in the previously uncharacterized Mad3-like domain of *S. pombe* Bub1p. This domain is necessary and sufficient for nuclear accumulation. In bub1-A78V mutant cells, Bub3p and Mad3p also fail to accumulate at kinetochores when the SAC is activated. The essential role of Bub1p in the nuclear accumulation of Bub3p and Mad3p in unperturbed interphase and mitotic cells is a function not previously reported for any other checkpoint protein. In contrast, Mad1p is not dependent on Bub1p for its proper localization, an observation unique to fission yeast.

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Note added in proof. Reagents and observations listed as "Hardwick, unpublished results" have now been published as Vanoosthuyse *et al.* (2004), Mol. Cell. Biol., *22*: 9786–9801.

REFERENCES

Basi, G., Schmid, E., and Maundrell, K. (1993). TATA box mutations in the *Schizosaccharomyces pombe nmt1* promoter affect transcription efficiency but not the transcription start point or thiamine repressibility. Gene *123*, 131–136.

Basu, J., Bousbaa, H., Logarinho, E., Li, Z., Williams, B. C., Lopes, C., Sunkel, C. E., and Goldberg, M. L. (1999). Mutations in the essential spindle checkpoint gene *bub1* cause chromosome missegregation and fail to block apoptosis in *Drosophila*. J. Cell Biol. *146*, 13–28.

Basu, J., Logarinho, E., Herrmann, S., Bousbaa, H., Li, Z., Chan, G. K., Yen, T. J., Sunkel, C. E., and Goldberg, M. L. (1998). Localization of the *Drosophila* checkpoint control protein Bub3 to the kinetochore requires Bub1 but not Zw10 or Rod. Chromosoma *107*, 376–385.

Bernard, P., Hardwick, K., and Javerzat, J. P. (1998). Fission yeast Bub1p is a mitotic centromere protein essential for the spindle checkpoint and the preservation of correct ploidy through mitosis. J. Cell Biol. *143*, 1775–1787.

Bernard, P., Maure, J. F., and Javerzat, J. P. (2001). Fission yeast Bub1p is essential in setting up the meiotic pattern of chromosome segregation. Nat. Cell Biol. *3*, 522–526.

Bharadwaj, R., and Yu, H. (2004). The spindle checkpoint, aneuploidy, and cancer. Oncogene *23*, 2016–2027.

Brady, D. M., and Hardwick, K. G. (2000). Complex formation between Mad1p, Bub1p and Bub3p is crucial for spindle checkpoint function. Curr. Biol. *10*, 675–678.

Campbell, M. S., Chan, G. K., and Yen, T. J. (2001). Mitotic checkpoint proteins HsMAD1 and HsMAD2 are associated with nuclear pore complexes in interphase. J. Cell Sci. *114*, 953–963.

Chen, R. H. (2002). BubR1 is essential for kinetochore localization of other spindle checkpoint proteins and its phosphorylation requires Mad1. J. Cell Biol. *158*, 487–496.

Chen, R. H., Shevchenko, A., Mann, M., and Murray, A. W. (1998). Spindle checkpoint protein Xmad1 recruits Xmad2 to unattached kinetochores. J. Cell Biol. *143*, 283–295.

Cleveland, D. W., Mao, Y., and Sullivan, K. F. (2003). Centromeres and kinetochores: from epigenetics to mitotic checkpoint signaling. Cell *112*, 407–421.

Demeter, J., Morphew, M., and Sazer, S. (1995). A mutation in the RCC1-related protein Pim1p results in nuclear envelope fragmentation in fission yeast. Proc. Natl. Acad. Sci. USA *92*, 1436–1440.

Ding, D. Q., Chikashige, Y., Haraguchi, T., and Hiraoka, Y. (1998). Oscillatory nuclear movement in fission yeast meiotic prophase is driven by astral microtubules, as revealed by continuous observation of chromosomes and microtubules in living cells. J. Cell Sci. *111*(Pt 6), 701–712.

Gillett, E. S., Espelin, C. W., and Sorger, P. K. (2004). Spindle checkpoint proteins and chromosome-microtubule attachment in budding yeast. J. Cell Biol. *164*, 535–546.

Hardwick, K. G., Johnston, R. C., Smith, D. L., and Murray, A. W. (2000). MAD3 encodes a novel component of the spindle checkpoint which interacts with Bub3p, Cdc20p, and Mad2p. J. Cell Biol. *148*, 871–882.

He, X., Jones, M. H., Winey, M., and Sazer, S. (1998). Mph1p, a member of the Mps1-like family of dual specificity protein kinases, is required for the spindle checkpoint in *S. pombe.* J. Cell Sci. *111*(Pt 12), 1635–1647.

He, X., Patterson, T. E., and Sazer, S. (1997). The *Schizosaccharomyces pombe* spindle checkpoint protein Mad2p blocks anaphase and genetically interacts with the anaphase-promoting complex. Proc. Natl. Acad. Sci. USA *94*, 7965–7970.

Howell, B. J., Hoffman, D. B., Fang, G., Murray, A. W., and Salmon, E. D. (2000). Visualization of Mad2 dynamics at kinetochores, along spindle fibers, and at spindle poles in living cells. J. Cell Biol. *150*, 1233–1250.

Howell, B. J., Moree, B., Farrar, E. M., Stewart, S., Fang, G., and Salmon, E. D. (2004). Spindle checkpoint protein dynamics at kinetochores in living cells. Curr. Biol. *14*, 953–964.

Hoyt, M. A., Totis, L., and Roberts, B. T. (1991). *S. cerevisiae* genes required for cell cycle arrest in response to loss of microtubule function. Cell *66*, 507–517.

Iouk, T., Kerscher, O., Scott, R.J., Basrai, M. A., and Wozniak, R. W. (2002). The yeast nuclear pore complex functionally interacts with components of the spindle assembly checkpoint. J. Cell Biol. *159*, 807–819.

Jablonski, S. A., Chan, G. K., Cooke, C. A., Earnshaw, W. C., and Yen, T. J. (1998). The hBUB1 and hBUBR1 kinases sequentially assemble onto kinetochores during prophase with hBUBR1 concentrating at the kinetochore plates in mitosis. Chromosoma *107*, 386–396.

Johnson, V. L., Scott, M. I., Holt, S. V., Hussein, D., and Taylor, S. S. (2004). Bub1 is required for kinetochore localization of BubR1, Cenp-E, Cenp-F and Mad2, and chromosome congression. J. Cell Sci. *117*, 1577–1589.

Kim, S. H., Lin, D. P., Matsumoto, S., Kitazono, A., and Matsumoto, T. (1998). Fission yeast Slp1p: an effector of the Mad2-dependent spindle checkpoint. Science *279*, 1045–1047.

Lew, D. J., and Burke, D. J. (2003). The spindle assembly and spindle position checkpoints. Annu. Rev. Genet. *37*, 251–282.

Li, R., and Murray, A. W. (1991). Feedback control of mitosis in budding yeast. Cell *66*, 519–531.

Maundrell, K. (1993). Thiamine-repressible expression vectors pREP and pRIP for fission yeast. Gene *123*, 127–130.

Millband, D. N., Campbell, L., and Hardwick, K. G. (2002). The awesome power of multiple model systems: interpreting the complex nature of spindle checkpoint signaling. Trends Cell Biol. *12*, 205–209.

Millband, D. N., and Hardwick, K. G. (2002). Fission yeast Mad3p is required for Mad2p to inhibit the anaphase-promoting complex and localizes to kinetochores in a Bub1p-, Bub3p-, and Mph1p-dependent manner. Mol. Cell. Biol. *22*, 2728–2742.

Moreno, S., Klar, A., and Nurse, P. (1991). Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe.* Methods Enzymol. *194*, 795–823.

Nair, R., Carter, P., and Rost, B. (2003). NLSdb: database of nuclear localization signals. Nucleic Acids Res. *31*, 397–399.

Ouyang, B., Lan, Z., Meadows, J., Pan, H., Fukasawa, K., Li, W., Dai, W. (1998). Human Bub1, a putative spindle checkpoint kinase closely linked to cell proliferation. Cell Growth Differ. *9*, 877–885.

Pasion, S. G., and Forsburg, S. L. (1999). Nuclear localization of *Schizosaccharomyces pombe* Mcm2/Cdc19p requires MCM complex assembly. Mol. Biol. Cell *10*, 4043–4057.

Sawin, K. E., and Snaith, H. A. (2004). Role of microtubules and Tea1p in establishment and maintenance of fission yeast cell polarity. J. Cell Sci. *117*, 689–700.

Shah, J. V., Botvinick, E., Bonday, Z., Furnari, F., Berns, M., and Cleveland, D. W. (2004). Dynamics of centromere and kinetochore proteins; implications for checkpoint signaling and silencing. Curr. Biol. *14*, 942–952.

Sharp-Baker, H., and Chen, R. H. (2001). Spindle checkpoint protein Bub1 is required for kinetochore localization of Mad1, Mad2, Bub3, and CENP-E, independently of its kinase activity. J. Cell Biol. *153*, 1239–1250.

Taylor, S. S., Ha, E., and McKeon, F. (1998). The human homologue of Bub3 is required for kinetochore localization of Bub1 and a Mad3/Bub1-related protein kinase. J. Cell Biol. *142*, 1–11.

Taylor, S. S., Hussein, D., Wang, Y., Elderkin, S., and Morrow, C. J. (2001). Kinetochore localisation and phosphorylation of the mitotic checkpoint components Bub1 and BubR1 are differentially regulated by spindle events in human cells. J. Cell Sci. *114*, 4385–4395.

Taylor, S. S., and McKeon, F. (1997). Kinetochore localization of murine Bub1 is required for normal mitotic timing and checkpoint response to spindle damage. Cell *89*, 727–735.

Tournier, S., Gachet, Y., Buck, V., Hyams, J., and Millar, J. (2004). Disruption of astral microtubule contact with the cell cortex activates a Bub1p, Bub3p, and Mad3p-dependent checkpoint in fission yeast. Mol. Biol. Cell *15*, 3345–3356.

Toyoda, Y., Furuya, K., Goshima, G., Nagao, K., Takahashi, K., and Yanagida, M. (2002). Requirement of chromatid cohesion proteins Rad21p/Scc1p and Mis4p/Scc2p for normal spindle-kinetochore interaction in fission yeast. Curr. Biol. *12*, 347–358.

Tran, P. T., Marsh, L., Doye, V., Inoue, S., and Chang, F. (2001). A mechanism for nuclear positioning in fission yeast based on microtubule pushing. J. Cell Biol. *153*, 397–411.

Warren, C. D., Brady, D. M., Johnston, R. C., Hanna, J. S., Hardwick, K. G., and Spencer, F. A. (2002). Distinct chromosome segregation roles for spindle checkpoint proteins. Mol. Biol. Cell *13*, 3029–3041.

Wassmann, K., and Benezra, R. (2001). Mitotic checkpoints: from yeast to cancer. Curr. Opin. Genet. Dev. *11*, 83–90.

Weiss, E., and Winey, M. (1996). The *Saccharomyces cerevisiae* spindle pole body duplication gene *MPS1* is part of a mitotic checkpoint. J. Cell Biol. *132*, 111–123.

Yamaguchi, S., Decottignies, A., and Nurse, P. (2003). Function of Cdc2p-dependent Bub1p phosphorylation and Bub1p kinase activity in the mitotic and meiotic spindle checkpoint. EMBO J. *22*, 1075–1087.

Yoshida, M., and Sazer, S. (2004). Nucleocytoplasmic transport and nuclear envelope integrity in the fission yeast *Schizosaccharomyces pombe.* Methods *33*, 226–238.

Yu, H. (2002). Regulation of APC-Cdc20 by the spindle checkpoint. Curr. Opin. Cell Biol. *14*, 706–714.